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Species Richness and Diversity of Gut Microbiota may Reduce AHPND in the Whiteleg Shrimp, *Litopenaeus vannamei*

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Acute hepatopancreatic necrosis disease (AHPND) causes massive economic loss in shrimp culture sector. Eco-friendly feed additives, which can reduce antibiotic resistance in bacteria and antibiotic residues present in aquaculture products, have become popular in shrimp farming. These additives have proven to maintain a balance host gut microbiota in preventing pathogen infection. In the present study, gut microbiota of *Litopenaeus vannamei*, which had been administrated with different diets (probiotic: *Bacillus* B2; prebiotic: *Gracilaria changii*, G; and a combination of *Bacillus* B2 and *G. changii*, B2+G) and post-challenged with AHPND-causing *Vibrio parahaemolyticus* strain 3HP, was determined using the PCR-DGGE method. The results showed that the gut microbiota of *L. vannamei* was dominated by the phylum Proteobacteria. The abundance of Proteobacteria was higher in the B2+G and G treatment groups. High abundance of native gut microbiota, such as Proteobacteria might prevent the adhesion of virulent bacteria by competitive exclusion, and thus, reducing the chance of AHPND infection. Moreover, the shrimp from the B2+G treatment group with higher survival in the challenge tests possessed a higher diversity and species richness of gut microbiota. This suggests that higher diversity and species richness of beneficial gut microbiota probably interfering bacterial communication among the pathogens by either destructing or neutralising their quorum sensing signals. Consequently, this will prevent the colonisation of pathogens in the gut and resulted in a higher survival of the shrimp challenged with *V. parahaemolyticus*.

Keywords: acute hepatopancreatic necrosis disease (AHPND); red seaweed; *Gracilaria changii*; *Bacillus*; gut microbiota; *Litopenaeus vannamei*

I. INTRODUCTION

Litopenaeus vannamei or the whiteleg shrimp is one of the most valuable cultured penaeid shrimp species in the world (GOAL, 2016; Thitamadee *et al.*, 2016) due to its tolerance to high-density cultivation (Cuzon *et al.*, 2004; FAO, 2004), and adaptability to wide ranges of environmental parameters, such as salinity and temperature (FAO, 2004; Pan *et al.*, 2007). The estimated production of *L. vannamei* had reached 77% out of total shrimp production in 2018 (GOAL, 2016; Thitamadee *et al.*, 2016). However, the intensification of the shrimp farming and improper farm management had led to the outbreak of shrimp diseases. Acute hepatopancreatic necrosis disease (AHPND) is one of the significant diseases, which caused mass mortalities of shrimp in Asian shrimp culture sector since 2009 (Tran *et al.*, 2013). AHPND is caused by *Vibrio* spp. that carried an extrachromosomal plasmid, pVA1, which encodes binary

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toxin gene, pirA/pirB^{vp} that destructs the hepatopancreas of the shrimp. These toxin genes are flanked between a set of transposable elements, which enables them to transfer between different species of *Vibrio* bacteria (Han *et al.*, 2015; Lee *et al.*, 2015). High density of the *Vibrio* in the culture environment might promote such transfer and result in the outbreak of AHPND. Although ingestion of AHPND-causing *V. parahaemolyticus* might be the natural route of infection of AHPND (Lee *et al.*, 2015), modulation of gut microbiota by feeding large amount of beneficial gut microbiota in the early life stages of shrimp will assist in the prevention of colonisation and infection of highly virulent pathogens such as AHPND-causing bacteria (Coutteau *et al.*, 2016).

Antibiotics have been extensively used to control bacterial disease outbreak previously. However, the use of antibiotics is not suitable for sustainable aquaculture and may cause the development of antibiotic resistance genes among host and in the environment (Baticados *et al.*, 1990; Cabello, 2006). In these few decades, the use of eco-friendly alternatives to antibiotics such as probiotics, prebiotics, seaweed, organic acids, yeast extracts, antimicrobial peptides and phytobiotics are getting popular among researchers and farmers in aquaculture field to replace the use of antibiotics (Amengyogbe *et al.*, 2020; Van Hai, 2015). From the literature surveys, most of the studies were reporting on the effects of probiotics and prebiotics on the physiological and immunological responses of the host against pathogens (Balcázar *et al.*, 2007; Chotigeat *et al.*, 2004; Immanuel *et al.*, 2012; Vaseeharan & Ramasamy, 2003), but limited studies have been focused on the effect of probiotics or prebiotics on the composition of indigenous microbiota of shrimp (Liu *et al.*, 2010; Luis-Villasenor *et al.*, 2015; Sha *et al.*, 2016). Several studies also demonstrated that modulation of the gut microbiota of *L. vannamei* has improved the growth, health and survival of the host (Suo *et al.*, 2017; Zhang *et al.*, 2014; Zhang *et al.*, 2016). For instance, Luis-Villaseñor *et al.* (2015) reported that *Bacillus* mixture (*B. tequilensis* YC5-2 + *B. endophyticus* C2-2 and YC3-B) was able to modulate the gut microbiota of *L. vannamei* and inhibit the growth of *Vibrio* sp.. Our previous study also showed that administration of the combined probiotic from gut of shrimp and the seaweed, *Gracilaria changii* was able to improve the survival rate of shrimp after challenged with AHPND-causing *V. parahaemolyticus* (Lim *et al.*, 2019). Hence, the present study aimed to determine the effects of *Bacillus* B2 obtained from the gut of *L. vannamei*; *G. changii*, and a seaweed-probiotic blend (combination of *Bacillus* B2 and *G. changii*) feed additives on the species diversity and species richness of the gut bacterial community in *L. vannamei* that post-challenged with AHPND-causing *V. parahaemolyticus*.

II. MATERIALS AND METHODS

A. Immersion Challenge Test of Shrimp against AHPND

The immersion challenge test was used to determine the synergistic antimicrobial effect of a seaweed-probiotic blend against AHPND-causing *V. parahaemolyticus* (Lim *et al.*, 2019). Briefly, three types of diets were compared in our previous study: commercial feed + *Bacillus* B2 (B2); commercial feed + *Bacillus* B2 fermented in 20 mg/g of *G. changii* (seaweed-probiotic blend/ B2+G); and commercial feed + *G. changii* (G). In addition, two control groups: the positive control group (commercial diet and challenged with *V. parahaemolyticus* strain 3HP) and the negative control group (commercial diet and not challenged with *V. parahaemolyticus* strain 3HP) were used in the study. The postlarvae of *L. vannamei* (PL16) used has an initial weight of 4.88 ± 0.71 mg. During the immersion test, an overnight culture of the AHPND-causing *V. parahaemolyticus* strain 3HP was prepared and added directly into each of the 1.2L plastic containers to reach a bacterial density of approximately 10^6 CFU/mL after 21 day of feeding trials. The survival of shrimps was monitored daily for the following 15 days. The shrimps, which survived at the end of the *in vivo* challenge tests, were collected, cleaned and stored at -20 °C for DNA extraction.

B. Extraction of DNA from Shrimp Guts

The shrimp from each treatment group and the control groups (n= 6) were prepared for the extraction of DNA. First, the body surface of shrimp was cleaned with 70% denatured ethanol and sterile cotton wool prior to extraction of DNA. The foregut, midgut and hindgut of each shrimp was excised using a pair of sterile forceps and scissors. The gDNA was

isolated from the samples according to the instructions provided in the DNeasy Blood & Tissue Kits (Qiagen, USA) with some pre-treatment procedures. Firstly, the excised intestinal tract was placed in a 1.5 µL microcentrifuge tube and ground with a sterile micropipette tip to squash the tissues. Then, 240 µL of 50 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 (consists of 18.01 g/L Na_2EDTA and 2.028 g/L NaOH that dissolved in ddH_2O), and 60 µL of 10 mg/mL lysozymes were added into the homogenates and incubated at 55°C for 45 min to lyse the cell wall of the gram-positive bacteria. Next, the samples were further processed according to the manufacturer's instruction (Qiagen, USA).

C. Polymerase Chain Reaction (PCR) Amplification for V3 Region of 16S rRNA Gene of Shrimp Gut Bacteria

PCR of variable 3 region (V3) of the 16S rRNA gene of all the bacteria presented in the intestinal samples of all the treated groups were conducted. The amplification of the V3 region was performed using the forward 338-F primer 5'-ACTCCTACGGGAGGCA-3' and reverse primer 518-R primer 5'-ATTACCGCGGCTGCTGG-3' (Rungrassamee *et al.*, 2013). In addition, a GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) was applied to the 5' end of the forward primer to increase the sensitivity of the DGGE analysis (Lim *et al.*, 2019; Muyzer & Smalla, 1998). PCR were then performed with an initial denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 30 s; annealing at 57°C for 30 s; elongation at 72°C for 30 s; and final elongation at 72°C for 5 min. Amplified products were analysed by electrophoresis in 4% agarose gels post-stained with ethidium bromide. Then, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen, USA) for denaturing gradient gel electrophoresis (DGGE).

D. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE of the PCR products generated with the 338F-GC/518R primer set were performed using the VS20WAVE-DGGE system (Cleaver Scientific Ltd, USA) according to the method described by Rungrassamee *et al.* (2013) with some modifications. Of which, the 10% of polyacrylamide gel (19:1 acrylamide/bisacrylamide) (Prakitchaiwattana *et al.*, 2004) that contained 55-80% urea and formamide were prepared. Approximately 600 ng of the PCR products of the three treatment groups, along with the positive and the negative control groups were loaded into their respective wells. The DGGE was run at 80V, 60°C for 18 h. The gel was then viewed using a gel imager (Syngene, UK) after it was stained with ethidium bromide. The DGGE analyses were performed for each treatment group and control group (n= 6).

E. Sequence Analysis

The qualitative DGGE results were further analysed with DNA sequencing. Each of the visible band observed on the DGGE gel was cut out, and extracted with FavorPrep™ GEL purification mini kit (FAVORGEN, Taiwan). The extracted product was re-amplified using the same sets of primers to get the optimum concentration for sequencing (Liu *et al.*, 2010). Then, all the re-amplified PCR product were outsourced for sequencing (Apical Scientific Sdn. Bhd., Malaysia). The 16S rRNA sequence of each species was determined and compared to the sequences deposited in the GenBank database using the similarity search program BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were compared with the sequences deposited in GenBank and the identity of each sequence was tabulated.

F. Biodiversity Analysis of Shrimp Gut Microbiota in Different Treatment Groups

The alpha diversity of the gut microbiota of different treatment groups were estimated by determining the Shannon's-index, and Simpson's diversity index. The species richness was determined by Margalef's index.

G. Statistical Analysis

All the statistical analysis was performed using the software package OriginPro 2017 (OriginLab Corporation, Northampton, Massachusetts, US). As the biodiversity indexes were not normally distributed, thus, Kruskal-Wallis test by ranks and postdoc Mann-Whitney U test were applied.

III. RESULTS

Gut microbiota of shrimp fed with different combination of additives were analysed by PCR-DGGE of the 16S rRNA V3 region in the present study. The band patterns in the DGGE polyacrylamide gel indicated that each of the experiment groups and the control groups (positive and negative controls) have different bacterial communities (Figure 1).

The identity of each specific bands represented by their relative abundance of each phylum were shown in Figure 2a, whereas the genus in Figure 2b. Generally, the most dominant bacterial groups, which were present in the guts of *L. vannamei* at phylum level were Proteobacteria (57.45%) and uncultured bacteria (36.52%). Among all the experiment groups, the abundance of Proteobacteria was higher in the G treatment group (80.00%) and the B2+G treatment group (20.00%) (Figure 2a). At the genus level, the gut microbiota of *L. vannamei* was dominated by *Ruegeria* sp. and *Maritimibacter* (Figure 2b). *Bacillus* spp. were observed in the positive control and the B2+G treatment group, with the abundance of 12.5% and 4%, respectively. However, *Bacillus* species was not found in the B2 groups. On the other hand, *Vibrio* sp. was only observed in B2+G treatment group, with the abundance of 8%. Surprisingly, the genus *Antarctobacter* was only found in the treated groups (B2 treatment, B2+G treatment and G treatment groups), and absence in both of the positive and negative control groups.

A. Alpha Diversity of the Gut Microbiota

Based on Shannon's index (H'), the gut microbiota of the G treatment and B2+G treatment groups showed a relatively higher alpha diversity as compared to the B2 treatment and control groups (Table 1). Whilst the alpha diversity of gut microbiota in the B2+G treatment group was the highest among the experiment groups based on Simpson's diversity index (1-λ). On the other hand, the B2+G treatment group has the highest species richness based on Margalef's index.

IV. DISCUSSION

In recent years, some studies proved that the manipulation of gut microbial composition has positive effect on the health, growth and survival of *L. vannamei* (Li *et al.*, 2018; Luis-Villasenor *et al.*, 2015; Sha *et al.*, 2016; Zhang *et al.*, 2014). The manipulation of gut microbiota can be affected by several factors such as genetic background, diet (Zhang *et al.*, 2014), and living environments of the host (Romero *et al.*, 2014). In addition, several studies had revealed that administration of prebiotics, probiotics or synbiotics is able to modulate or maintain the balanced gut microbiota of shrimp, and thus preventing the adhesion of opportunistic pathogens (Jha *et al.*, 2016; Luis-Villasenor *et al.*, 2013; Luis-Villasenor *et al.*, 2015; Sha *et al.*, 2016). Several

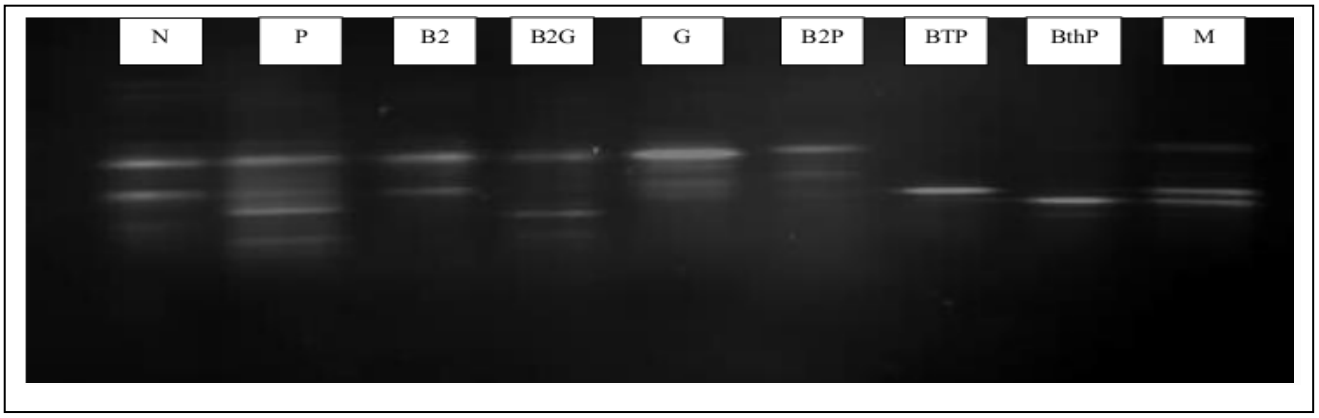

Figure 1. Denaturing gradient gel electrophoresis (DGGE) (8% of 19:1 acrylamide: bisacrylamide) gel profiles of the V3 region amplified by PCR of the DNA extracted from the gut of the shrimp treated with different test diets. N- negative control; P- positive control; B2- B2 isolates; B2G- combination of B2 and 20 mg/mL of *Gracilaria changii*; G- 20 mg/mL of *Gracilaria changii*; B2P- positive control of B2; BTP- positive control of *B. subtilis* BT; BthP- *B. thuringiensis* ATCC strain; R- references by mixing B2P, BTP and BthP.

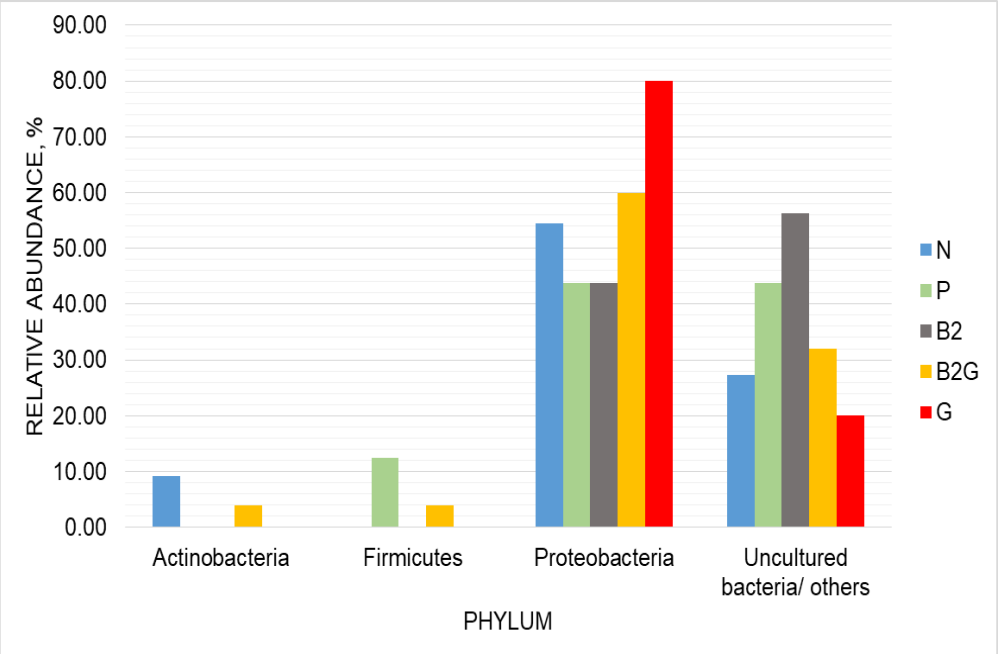

Figure 2a. Relative abundance of bacteria (Phylum) in the gut of *Litopenaeus vannamei* from different treatment groups. N-negative control; P- positive control; B2- B2 isolates; B2G- Combination of B2 and 20 mg/ml of *Gracilaria changii*; and G- 20 mg/ml of *G. changii*.

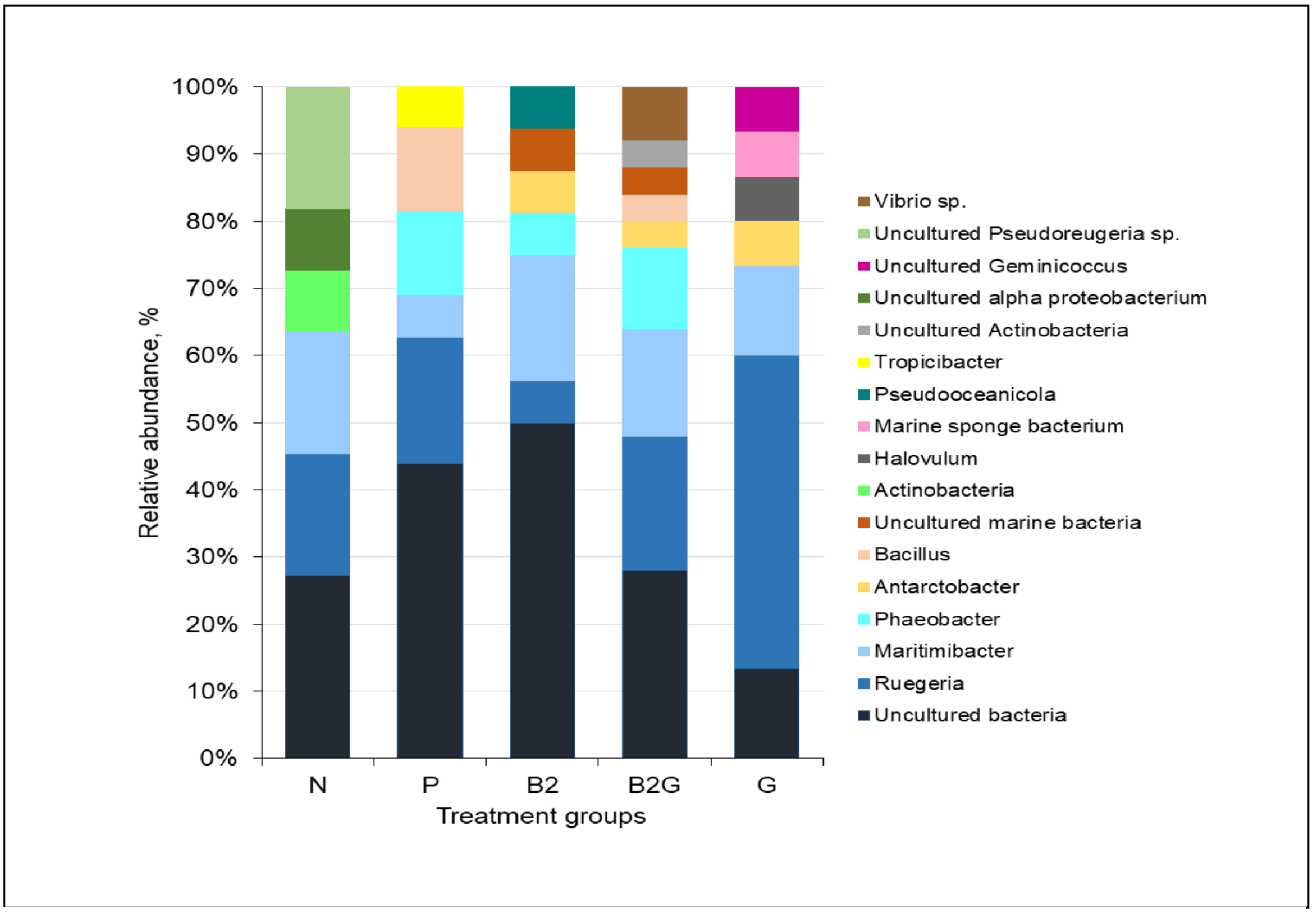

Figure 2b. Relative abundance of bacteria (genera) in the gut of *Litopenaeus vannamei* from different treatment groups. N-negative control; P- positive control; B2- B2 isolates; B2G- Combination of B2 and 20 mg/ml of *Gracilaria changii*; and G- 20 mg/ml of *G. changii*.

researchers have also suggested administration of natural feed additives as one of the possible ways to prevent AHPND (Jha *et al.*, 2016; Wang *et al.*, 2018). Our previous study had showed that the administration of the seaweed-probiotic

Table 1. Biodiversity analysis of the gut microbiota of *Litopenaeus vannamei* in different experiment groups. N-negative control; P- positive control; B2- B2 isolates; B2G- Combination of B2 and 20 mg/ml of *Gracilaria changii*; and G- 20 mg/ml of *G. changii.*

Experimental groups	No. of DGGE bands	Shannon index (H')	Simpson's dominance index (D)	Simpson's diversity index (1-λ)	Margalef's index
N	11	1.72	5.26	0.81	5.04
P	16	1.37	3.82	0.74	5.17
B2	16	1.53	3.28	0.70	6.17
B2G	25	1.91	6.32	0.84	8.28
G	15	3.23	3.69	0.69	6.15

blend (combination of *Bacillus* B2 and *G. Changii*) significantly improved the survival of *L. vannamei* challenged with AHPND-causing bacteria (Lim *et al.*, 2019). The present study was the first report that showed the positive effect of the seaweed-probiotic blend as feed additives on the gut microbiota modulation of *L. vannamei*, against the challenge with AHPND-causing *V. parahaemolyticus* strain 3HP.

Proteobacteria was the most dominant member in the gut microbiota of *L. vannamei* at phylum level based on our result. This result concurs with previous studies which reported that Proteobacteria was the most prevalent member in shrimp (Md Zoqratt *et al.*, 2018; Rungrassameeet *et al.*, 2013; Sha *et al.*, 2016; Zhang *et al.*, 2016). Moreover, Proteobacteria was considered as the most stable gut-associated microorganisms in shrimp because studies showed that their abundance did not vary in conditions such as salinity of the water (Zhang *et al.*, 2016), sulfide exposure (Suo *et al.*, 2017), and diet content (Qiao *et al.*, 2016; Zhang *et al.*, 2014). Besides, the abundances of Proteobacteria were higher in the B2+G and G treatment groups, which showed higher survival rate in shrimp that post-challenged with AHPND-causing *V. parahaemolyticus* in our previous study (Lim *et al.*, 2019). Hence, this suggests that Proteobacteria can be the native gut microbiota, which potentially inhibits the growth of opportunistic pathogens by competitive exclusion principles such as competition for space and resources (Lio-Po *et al.*, 2005; Natrah *et al.*, 2011). At the genus level, the gut microbiota of *L. vannamei* was dominated by uncultured bacteria, *Ruegeria* sp. and *Maritimibacter*. *Ruegeria* is a genus of bacteria under the phylum Proteobacteria, which has been widely used as a probiotic in aquaculture (Barreto-Curiel *et al.*, 2018). It is also proved to have antimicrobial effect against *V. anguillarum* and improved the survival of Atlantic cod larvae (Fjellheim *et al.*, 2010).

On the other hand, *Bacillus* sp. was not detected in the experiment groups fed with *Bacillus* B2. This indicated that *Bacillus* B2 did not efficiently colonise and establish on the gut of the shrimp or their abundance in the gut was too low to be detected. Landsman *et al.* (2019) also revealed that the abundance of administrated probiotics was low or not detected in the gut of the shrimp, but the presence of these probiotics probably modulate the gut bacterial communities. They hypothesised that even if the probiotics was not established at a high density in the gut of the hosts, they might produce metabolites, which favour the establishment of certain bacterial species in the gut of the shrimp (Landsman *et al.*, 2019). Interestingly, the presence of *Antarctobacter* in the shrimp from the B2, B2+G and G treatment groups and its absence in both the control groups suggest that these feed additives probably encourage the growth of *Antarctobacter* in the shrimp guts. Some studies also showed that *Antarctobacter* can possibly be used as antimicrobial agents against marine pathogens. For instance, Høj *et al.* (2009) showed that enrichment of artemia with microalgae led to the detection of *Antarctobacter* spp. in artemia. Sharifah and Eguchi (2011) showed that the combination of the phytoplankton, *Nannochloropsis oculata* and the probiotic, *Antarctobacter* sp. are able to exert the synergistic effect against fish pathogen, *V. anguillarum*. Therefore, this indicates that *Antarctobacter* found in the gut microbiota of the treated groups in the present study probably have beneficial effect towards the

shrimp in order to prevent the colonisation of pathogens. However, further investigations are required to determine the effectiveness of *Antarctobacter* sp. as probiotics in aquaculture industry.

For the alpha diversity of the gut microbiota, a low value of Shannon's index suggests that the shrimp fed with B2 alone in the present study had a lower bacterial diversity as compared to the negative control group. Similarly, Sha *et al.* (2016) also found that the probiotic supplementation reduced the diversity of the gut microbiota of *L. vannamei*. However, Luis-Villasenor *et al.* (2013; 2015) showed that the probiotic mixture, commercial probiotic Alibio® and *Bacillus* mixture increase the bacterial diversity and evenness in the gut of shrimp. These probiotics also improved the survival of shrimp that challenged with the pathogens. Besides, Mazón-Suástegui *et al.* (2020) also showed that shrimp that fed with *Streptomyces* have higher gut bacterial diversity and greater tolerance to colonisation *V. parahaemolyticus* as compared to the control group. However, the underlying mechanisms for this protection against the pathogen infection remained unclear.

Similarly, the shrimp present in the seaweed-probiotic blend (B2+G) treatment group showed higher survival after challenged with AHPND-causing bacteria (Lim *et al.*, 2019), and has higher species richness as well as higher species diversity of the gut bacteria as compared to the control groups based on the Margalef's index and Shannon's indices. The seaweed-probiotic blend potentially increased the diversity of the unculturable gut microbiota in the shrimp (Landsman *et al.*, 2019). Hence, it can be hypothesised that higher diversity of microbial composition in the gut might be able to interfere the quorum sensing of pathogens by degrading or neutralising the signal molecules of pathogens (Defoirdt *et al.*, 2004). In addition, they are likely to produce some metabolites that can prevent the establishment of the pathogens, and thus reduce the chances of AHPND infection. However, the mechanisms of interaction between the shrimp gut microbiota and the opportunistic pathogens in the present study remained unclear. Future research is required to be carried out in order to determine the underlying mode of action of these gut microbiota in preventing AHPND infection.

V. CONCLUSION

In conclusion, the present study showed that the shrimp fed with different feed additives possess different composition of gut microbiota. Proteobacteria is the dominant phylum in the guts of *L. vannamei* of all the experiment groups. The present study also revealed that administration of the seaweed-probiotic blend (*Bacillus* B2 and *G. changii*) increases the diversity and species richness of the gut microbial composition. Moreover, higher abundance of Proteobacteria, which is a stable bacterial phylum commonly found in the gut of aquatic animals, in the B2+G treatment group may be one of the factors that prevents the invasion of highly virulent pathogens such as AHPND-causing *V. parahaemolyticus*. The presence of B2+G in the shrimp feed may provide a stable microbial community, which can potentially inhibit the growth of pathogens by competing for space and nutrient.

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